

or derivatives thereof, can be chemically synthesized (*See, e.g.*, Clark-Lewis et al., 1991, *Biochem.* 30:3128-3135 and Merrifield, 1963, *J. Amer. Chem. Soc.* 85:2149-2156). For example, the chimeric proteins disclosed in Section 4.2., or fragments, derivatives and analogues can be synthesized by solid phase techniques, cleaved from the resin, and  
5 purified by preparative high performance liquid chromatography (*e.g.*, see Creighton, 1983, *Proteins, Structures and Molecular Principles*, W.H. Freeman and Co., N.Y., pp. 50-60). The chimeric protein disclosed in Section 4.2., or fragment, derivatives and analogues can also be synthesized by use of a peptide synthesizer. The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (*e.g.*, the  
10 Edman degradation procedure; *see* Creighton, 1983, *Proteins, Structures and Molecular Principles*, W.H. Freeman and Co., N.Y., pp. 34-49).

The chimeric proteins disclosed in Section 4.2., or fragments, derivatives and analogues thereof, may be synthesized in their entirety by the sequential addition of amino acid residues or alternatively as fragment subcomponents which may be combined using  
15 techniques well known in the art, such as, for example, fragment condensation (Shin et al., 1992, *Biosci. Biotech. Biochem.* 56:404-408; Nyfeler et al., 1992, *Peptides*, Proc. 12th Amer. Pep. Soc., Smith and Rivier (eds), Leiden, pp 661-663; and Nokihara et al., 1990. Protein Research Foundation, Yanaihara (ed), Osaka, pp 315-320).

In a less preferred embodiment, the chimeric proteins disclosed in Section 4.2., or  
20 fragments, derivatives and analogues thereof, can be obtained by proteolysis of the protein followed by purification using standard methods such as those described above (*e.g.*, immunoaffinity purification).

#### **4.4. OBTAINING CORRECTLY FOLDED INSULIN PRECURSOR**

25 The present invention provides a process for obtaining a correctly folded first insulin-precursor-containing chimeric protein comprising, contacting an incorrectly folded second insulin-precursor-containing chimeric protein, which said second insulin-precursor-containing chimeric protein consists of an intramolecular chaperone (IMC) like peptidyl fragment separated from the insulin precursor by one or more cleavable amino acid residues, with at least one chaotropic auxiliary agent in an aqueous medium; wherein  
30 said IMC like peptidyl fragment: a) contains from about 20 to about 200 amino acid residues; b) is not the insulin precursor or a portion thereof; and c) improves the insulin precursor folding such that the yield of the correctly folded first insulin-precursor-containing chimeric protein when the incorrectly folded second insulin-precursor-containing chimeric protein is contacted with the chaotropic auxiliary agent is higher than  
35 the yield of the correctly folded insulin precursor when the incorrectly folded insulin

precursor, which does not contain said IMC like peptidyl fragment, is contacted with the same chaotropic auxiliary agent.

As used herein, the term "human insulin precursor" refers to a molecule which 1) contains the human insulin A chain and B chain, or analogues, derivatives and fragments 5 thereof, 2) contains six cysteine residues, 3) has a removable connecting moiety which is joined to the insulin A chain and B chain, and 4) is capable of being bound by an anti-human-insulin antibody. Examples of human insulin precursor include, but are not limited to, the ones disclosed in Ladisch and Kohlmann, *Biotechnol. Prog.*, 1992, 8:469-478; Thim et al., *Proc. Natl. Acad. Sci. USA*, 1986, 83:6766-6770; U.S Patent No. 10 5,473,049; U.S Patent No. 5,457,066; and EP 0,347,781 B1.

The term "correctly folded" human insulin precursor or insulin-precursor-containing chimeric protein refers to a molecule wherein the human insulin precursor has the conformation and disulfide bridges as found in a natural, biologically active human insulin, i.e., the disulfide bridges between a) A-6 and A-11, b) A-7 and B-7, c) A-20 and 15 B-19, are formed. The term "incorrectly folded" human insulin precursor or insulin-precursor-containing chimeric protein refers to a molecule wherein the human insulin precursor lacks the conformation, disulfide bridges as found in a natural, biologically active human insulin, or both.

In a preferred embodiment, the present invention provides a process for obtaining a 20 correctly folded insulin-precursor-containing chimeric protein described above, wherein the insulin precursor is of human origin. Also preferable, the human insulin precursor is capable of being bound by an anti-human-insulin antibody. Still preferably, the human insulin precursor consists of the amino acid sequence of SEQ ID NO:4. Yet preferably, in the human insulin precursor, B chain and A chain of the human insulin precursor are 25 separated by an amino acid residue or a peptidyl fragment consisting of 2 to 34 amino acid residues. More preferably, the human insulin precursor consists of the amino acid sequence of SEQ ID NO:5.

In a preferred embodiment, the present invention provides a process for obtaining a correctly folded insulin-precursor-containing chimeric protein described above, wherein 30 the IMC like peptidyl fragment contains higher percentage of charged amino acid residue than the insulin precursor. Also preferably, wherein in the IMC like peptidyl fragment, the N-terminal half contains more positively charged amino acid residues than negatively charged amino acid residues and the C-terminal half contains more negatively charged amino acid residues than positively charged amino acid residues. Still preferably, the 35 IMC like peptidyl fragment consists of an amino acid sequence that has at least 40% identity to a domain containing at least first 20 N-terminal amino acids of human growth

hormone (hGH) protein, in which the percentage identity is determined over an amino acid sequence of identical size to the domain of hGH. Yet preferably, the IMC like peptidyl fragment consists of an amino acid sequence that has at least 60% identity to a domain containing at least first 20 N-terminal amino acids of human growth hormone (hGH)

5 protein. Yet preferably, the IMC like peptidyl fragment is capable of being bound by an anti-hGH antibody. More preferably, the IMC like peptidyl fragment consists of the amino acid sequence of SEQ ID NO:1. Also more preferably, the IMC like peptidyl fragment consists of the amino acid sequence of SEQ ID NO:2.

In a preferred embodiment, the present invention provides a process for obtaining a correctly folded insulin-precursor-containing chimeric protein described above, wherein the cleavable amino acid residue is an Arg or a Lys residue. Also preferably, the cleavable amino acid residues consist of the amino acid sequence of SEQ ID:3.

In a specific embodiment, the present invention provides a process for obtaining a correctly folded insulin-precursor-containing chimeric protein described above, wherein in 15 the incorrectly folded second insulin-precursor-containing chimeric protein, the IMC like peptidyl fragment is located at the N-terminus of said chimeric protein. In another such specific embodiment, the IMC like peptidyl fragment is located at the C-terminus of said chimeric protein. In still another such specific embodiment, the IMC like peptidyl fragment is located between the B chain and A chain of the insulin precursor.

20 In a preferred embodiment, the present invention provides a process for obtaining a correctly folded insulin-precursor-containing chimeric protein described above, wherein the IMC like peptidyl fragment contains one or more cleavable amino acid residues which are identical to the one or more cleavable amino acid residues that separate the IMC like peptidyl fragment and the insulin precursor in the second insulin-precursor-containing 25 chimeric protein. More preferably, the cleavable amino acid residue is an Arg or a Lys residue.

In a most preferred embodiment, the present invention provides a process for obtaining a correctly folded insulin-precursor-containing chimeric protein described above, wherein the incorrectly folded second insulin-precursor-containing chimeric protein 30 consists of the amino acid sequence of SEQ ID NO:6. Also most preferably, the incorrectly folded second insulin-precursor-containing chimeric protein consists of the amino acid sequence of SEQ ID NO:7.

Chaotropic auxiliary agents are compounds which break hydrogen bonds in aqueous solution. In a specific embodiment, the present invention provides a process for obtaining 35 a correctly folded insulin-precursor-containing chimeric protein described above, wherein the chaotropic auxiliary agent is selected from the group consisting of guanidine